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Cross-protection of pepper plants stressed by copper against a vascular pathogen is accompanied by the induction of a defence response

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ABSTRACT

Pepper (*Capsicum annuum* L.) plants stressed by copper showed less disease symptoms after inoculation with *Verticillium dahliae* Kleb. We tested if such protection was accompanied of a defence response induced by copper stress by measurement of peroxidase and chitinase activity, phenolics and the expression of four genes related to plant defence. Peroxidase activity, but not chitinase, increased in roots, stem and leaves of copper-stressed plants. However, treating the plants with an ethylene perception inhibitor (MCP) before applying the copper stress, caused a synergic enhancement of both enzymes in stem and cotyledons. Phenolic compounds were also induced by copper but downregulated by MCP in stem. The expression of a peroxidase gene (*CAPO1*), a sesquiterpene cyclase gene (*CASC1*), a PR1 gene (*CABPR1*) and a β-1,3-glucanase (*CABGLU*) was highly upregulated by copper stress, but MCP neither suppresses nor enhances such an effect. Globally, copper stress causes an induction of defence mechanisms that may partially explain tolerance to Verticillium wilt.

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1. Introduction

Heavy metals are sometimes present in phytotoxic amounts in soils as a result of agricultural and industrial activities [1]. Excessive uptake of such metals by the plants may eventually affect different physiological processes. For example, copper sometimes reaches high levels in the soil because of mining activities or the prolonged application of copper-based fungicides, such as Bordeaux mixture, in old orchards and vineyards [1,2]. In plants, copper stress inhibits photosynthesis, respiration and nitrogen fixation, and causes the alteration of membrane integrity, the formation of active oxygen species and the subsequent enhancement of lipid peroxidation [2–4]. Alteration of processes at the cellular level leads to several macroscopic symptoms in plants suffering from copper stress, such as stunted growth, necrosis, leaf epinasty, chlorosis and red-brownish discoloration [5].

Heavy metals are not the only cause of plant stress. In nature, plants have to cope with various environmental conditions that differ from optimal conditions and they have to respond to different biotic and abiotic signals by adapting their development. Indeed, the exposure of plants to heavy metals may lead to protection against pathogens [6]. There are several explanations for this effect. Firstly, heavy metals are themselves toxic to pathogens, therefore metal accumulation by the plant may suppress pathogen infection. Secondly, heavy metals can act as elicitors of plant defence mechanisms [6,7].

Plants possess structural and biochemical mechanisms for defence against pathogens. One of the structural barriers that prevent plant colonization by pathogens is lignin, which is synthesized by peroxidases from cinnamyl alcohols [8]. Peroxidases and lignification are induced in plants by heavy metal stress [9–11] as well as after infection by pathogens [12]. Plants may also defend themselves against pathogens through the so-called "biochemical" defences, which normally include secondary metabolites (phytoanticipins and phytoalexins) and defence proteins. Many phytoanticipins and phytoalexins are phenolics or isoprenoids, and some of them are accumulated in response to heavy metal stress [13]. Likewise, other defence proteins such as PR proteins are induced by heavy metal stress [14,15].

Responses to both biotic and abiotic stress are mediated by low-molecular weight molecules, such as reactive oxygen species (ROS), salicylic acid, jasmonic acid, abscisic acid and ethylene [7,16,17]. These signals regulate the protective responses of plants against different stresses via synergistic and antagonistic actions, which are referred to as signalling crosstalk [16]. There is evidence

Abbreviations: MCP, 1-methylcyclopropene; MeOH, methanol; PCR, polymerase chain reaction; PVPP, polyvinylpolypyrrolidone; ROS, reactive oxygen species; SAR, systemic acquired resistance.

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of such crosstalk between ROS and jasmonic acid or other oxylipins in biotic and heavy metal stress [7,13]. A well-known signal regulating the so-called systemic acquired resistance (SAR) against pathogens, salicylic acid, has been reported to alleviate the negative effect of cadmium on barley [18] and maize plants [19]. However, the actual role of salicylic acid in response to plant abiotic stress is still unresolved [17]. Jasmonic acid and ethylene have also been related to response to heavy metal stress [20–22]. In fact, heavy metal stress caused by copper stimulates the biosynthesis of ethylene [23–25], which may act as an endogenous signal triggering the plant response to such stress. Ethylene is released from the plant in the response of plant to both biotic and abiotic stress [26]. In summary, plant response to pathogens and to abiotic stress, particularly the one caused by heavy metals, employ a lot of common signals and a cross-protection would be possible.

In a previous report, we showed that an excess of copper causes stress in pepper plants, inducing several physiological responses [9]. In the present study, we investigated the ability of copper stress to protect pepper plants against a plant disease, Verticillium wilt, as well as some of the plant defence mechanisms against pathogens that could be triggered by the exposure to such stress.

2. Materials and methods

2.1. Plant material, growth conditions and treatment procedures

Seeds of pepper (*Capsicum annuum* L.) were germinated in perlite, and seedlings were grown for 3–4 days after emergence and then used in the experiments described below. For all experiments, plants were grown at 25 °C under a 16-h photoperiod (Lamps OSRAM L 18W/765; 228 μmol m^{-2} s^{-1} PAR).

For the experiments of inoculation with *Verticillium dahliae* Kleb., a control group of plants was grown in perlite soaked in a nutrient solution composed of 6 mM KNO_3, 4 mM $Ca(NO_3)_2$, 2 mM $NH_4H_2PO_4$, 1 mM $MgSO_4$, 50 μM KCl, 25 μM H_3BO_3, 2 μM $MnSO_4$, 2 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.5 μM H_2MoO_4, 20 μM EDTA and 20 μM $Fe(NH_4)_2(SO_4)$. A second group was grown in the same nutrient solution but supplemented with 50 μM $CuSO_4$. The solutions were periodically renewed and aerated. Four days after these treatments, roots of plants were washed in distilled water and plants were challenge-inoculated with *V. dahliae* (isolate UDC53Vd) by dipping the roots in a suspension of 10^6 conidia ml^{-1} for 45 min [27]. The control groups were inoculated with water. Following inoculation, the plants were transferred to pots containing a mixture of perlite and potting soil (1:2, v/v). The severity of Verticillium wilt symptoms was estimated both by the reduction of the length of the stem in relation to non-inoculated plants and the percentage of wilted leaves per plant. Both parameters were monitored weekly until 28 days after inoculation. The experiments were performed three times.

In a second set of experiments, a group of plants was exposed to 1-methylcyclopropene (MCP), an inhibitor of ethylene perception. Plants were exposed to MCP at a final concentration of 0.2 μL L^{-1} in a sealed container [28]. Control plants were kept in a container with no chemical added. Containers were opened after 8 h and plants were then treated with the control nutrient solution or with the 50 μM $CuSO_4$ supplemented solution described above. Samples of cotyledons, stems and roots were taken at 96 h after the beginning of the copper stress treatment and stored at −80 °C for further analysis. The experiments were performed at least twice for each parameter analysed (enzymes, gene expression, phenolics).

2.2. Enzyme extraction and assays

Cotyledons, stems or roots from 20 plants (0.3–1 g) were homogenised at 4 °C in 50 mM Tris–HCl buffer (pH 7.5) with the addition of 0.05 g polyvinylpolypyrrolidone (PVPP) per gram of fresh weight. Crude extracts were centrifuged at 10,000 × g at 4 °C for 20 min. Supernatants were desalted in a PD-10 column (GE Healthcare) and the eluate analysed for enzyme activity. Peroxidase activity was determined according to [27] and chitinase activity was determined by the method reported in [29]. Proteins were determined as in [27].

2.3. Extraction and determination of soluble phenolics

Stems from 20 plants (0.2–0.4 g) were homogenised in 2.5 ml of 80% MeOH. The homogenised sample was incubated for 15 min at 70 °C and then filtered. The residue in the filter was washed with 2.5 ml of 80% MeOH to optimise the extraction. The final volume was adjusted to 5 ml and used immediately for phenolic determination.

Total soluble phenols were determined with Folin–Ciocalteu reagent as described in [27]. The content of the soluble phenols was calculated from a standard curve obtained with different concentrations of gallic acid.

2.4. RNA extraction and cDNA synthesis

Total RNA was extracted from frozen samples with the Aurum™ Total RNA Mini Kit (BioRad), according to the manufacturer's instructions. RNA quantity was measured spectrophotometrically and its integrity was checked by 1.2% agarose-formaldehyde gel electrophoresis. First strand cDNA was synthesized from 100 ng total RNA with the iScript cDNA Synthesis Kit (BioRad) and following the protocol supplied by the manufacturer.

2.5. Real-time RT-PCR assay

The expression of several genes related to defence against pathogens was studied. The genes were a peroxidase gene (*CAPO1*), a sesquiterpene cyclase gene (*CASC1*), a PR1 gene (*CABPR1*) and a β-1,3-glucanase (*CABGLU*). An actin gene (AY572427) was used as a constitutively expressed endogenous control, whose expression levels were essentially constant in the Cu conditions assayed. All the primers and gene accessions are described in [30]. Real-time PCR was performed in 50 μl of reaction mixture composed of 2.5 μl of cDNA, 1× iQ SYBR Green Supermix (BioRad) and 0.3 μM of each gene-specific primer, with an iCycler iQ system (BioRad). The thermal cycling conditions consisted of initial denaturation at 95 °C for 2 min followed by 40 cycles at 95 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min, and a final step at 72 °C for 5 min. The specificity was tested by identification of only one peak in the melting curve analysis. A fivefold series of dilutions of reverse transcribed total RNA concentrations was used to calculate the PCR reaction efficiency as described by Pfaffl [31]. This method defines the efficiency as the slope of the line formed by representation of the cycle thresholds (Ct) versus concentrations of the serial dilutions. The relationship between slope and efficiency is given by the equation: $E = 10^{-1/\text{slope}}$. The relative expression level of each gene used here depends on this efficiency and is described as the difference between the studied gen (target) Ct of the control and that of the sample, and later normalization with the reference gen (actin). The difference in Ct is the number of cycles that the amount of sample RNA needs to equal the amount of control RNA, therefore the relative expression is defined as follows: relative expression $= E^{\Delta Ct_{\text{target}}(\text{Control-sample})} / E^{\Delta Ct_{\text{reference}}(\text{Control-sample})}$. The relative expression is, therefore, the number of times that the amount of RNA template sample is higher or lower than the amount in the control and therefore, this level must be relative to the control level, taken as a standard value "1". Each experiment was repeated twice and each measurement was performed in duplicate.

2.6. Statistical analysis

All statistical analyses were performed with Statgraphics Plus for Windows, 5.1 Professional Version (Statistical Graphics Corp). When necessary, transformations were carried out to normalize the data prior to analysis. The Verticillium wilt data were compared by student t test ($p < 0.05$). In the rest of the experiments a one-way ANOVA was performed ($p < 0.05$) followed by Duncan test for multiple comparisons. Statistically significant differences ($\alpha = 0.05$) are reported in the text and shown in the figures.

3. Results

3.1. Cross-protection of Verticillium wilt of pepper by copper stress treatment

Previous experiments established that the copper concentration (50 μM $CuSO_4$) and the time of exposure (96 h) that we have applied in the present paper caused macroscopical symptoms of stress [9]. In the experiments reported here, the influence of copper stress on Verticillium wilt was assessed by measuring the reduction of the length of the stem with respect to non-inoculated plants and the percentage of wilted leaves. Three weeks after inoculation, the plants treated with copper showed a significantly ($p < 0.05$) lower reduction in the length of stem than the non-treated plants, and the difference was even more evident at the end of the assay (Fig. 1a). There were no wilted leaves, another Verticillium disease symptom, till the third week after inoculation. Both 21 and 28 days after inoculation copper-treated plants showed a significant ($p < 0.05$) lower number of wilted leaves than plants not treated with the metal (Fig. 1b).

3.2. Effect of copper stress on the defence response of pepper plants

Peroxidase activity was determined in a second set of experiments (Fig. 2). A significant increase in activity was observed in roots (Fig. 2a), stem (Fig. 2b) and cotyledons (Fig. 2c) of copper-treated plants. Blockage of ethylene perception by MCP also increased peroxidase activity in roots and cotyledons (Fig. 2a,c), but there were no significant differences in the case of stem (Fig. 2b). Interestingly, application of both MCP and copper had a synergic effect on the induction of peroxidase activity in both stem and cotyledons (Fig. 2b,c).

In the case of chitinase activity (Fig. 3), we did not observed a clear increase in the copper-treated plants in any organ, and the MCP only caused an increase in activity in the roots (Fig. 3a). However, a synergic effect of induction was observed in stems and

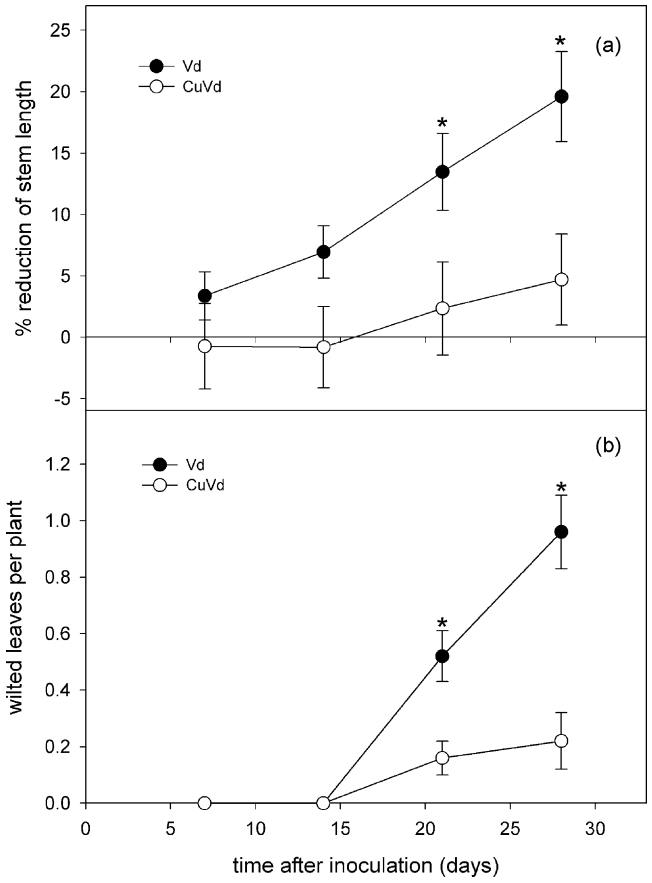

Fig. 1. Copper stress treatment protects pepper plants against Verticillium wilt. Values are means ± 1S.E. of three independent experiments ($n = 23$ per treatment). (a) Reduction in stem length respect to the control (b) wilted leaves. Vd-plants inoculated with *Verticillium dahliae*; CuVd-plants stressed with copper and inoculated with *Verticillium dahliae*. An asterisk (*) indicates statistically significant differences between both groups ($\alpha = 0.05$).

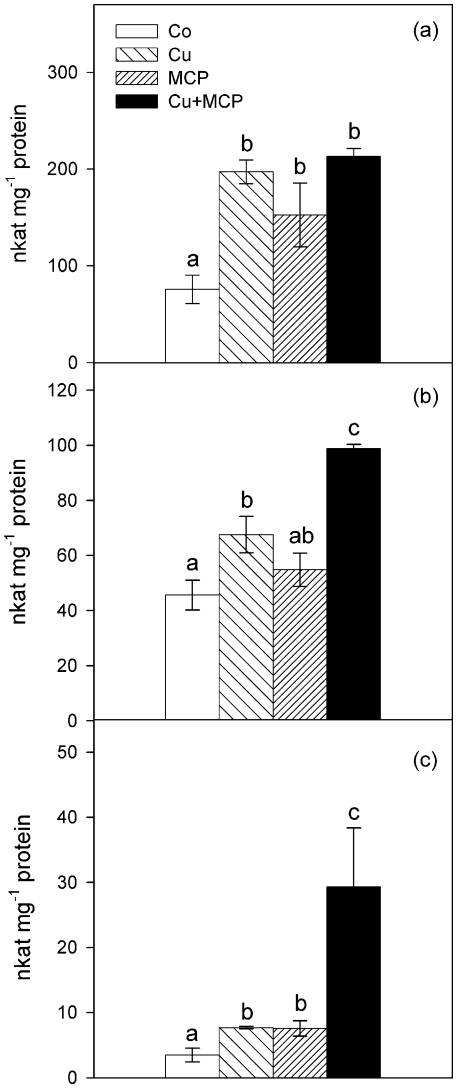

Fig. 2. Effect of copper stress and inhibition of ethylene perception on specific peroxidase activity in pepper plants. Values are means ± 1S.E. of three independent experiments. (a) Roots, (b) stem, and (c) Cotyledons. Co = Control; Cu = 50 μM $CuSO_4$; MCP = 0.2 μL L^{-1} 1-methylcyclopropene; Cu + MCP = 0.2 μL L^{-1} 1-methylcyclopropene + 50 μM $CuSO_4$. The letters above the bars indicate statistically significant differences between groups ($\alpha = 0.05$).

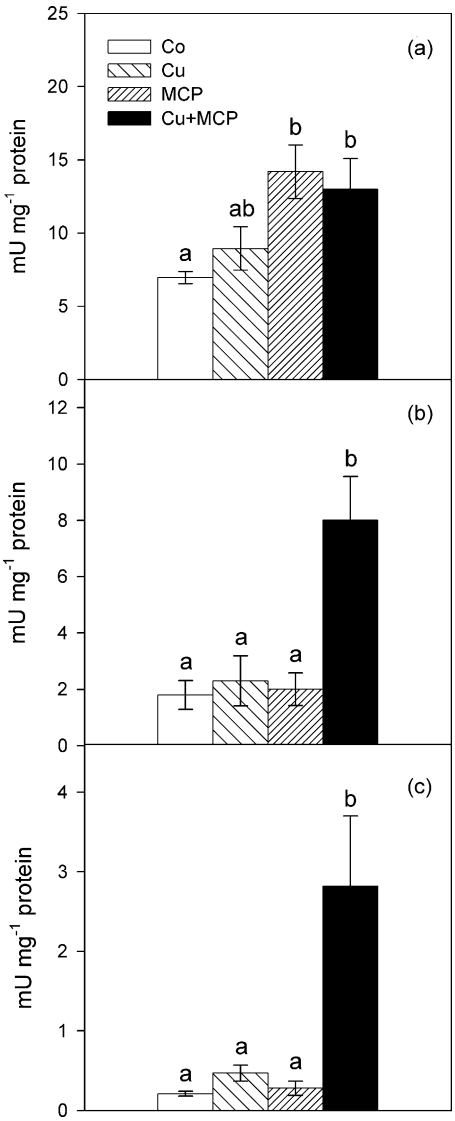

Fig. 3. Effect of copper stress and inhibition of ethylene perception on specific chitinase activity in pepper plants. Values are means ± 1S.E. of three independent experiments. (a) Roots, (b) stem, and (c) cotyledons. Co = Control; Cu = 50 μM $CuSO_4$; MCP = 0.2 μL L^{-1} 1-methylcyclopropene; Cu + MCP = 0.2 μL L^{-1} 1-methylcyclopropene + 50 μM $CuSO_4$. The letters above the bars indicate statistically significant differences between groups (α = 0.05).

cotyledons when MCP was applied and the plants were copper-stressed (Fig. 3b,c).

Because synergism was observed for both peroxidase and chitinase in stems, and taking into account that vascular tissues of this organ are usually affected by *Verticillium* infection, we decided to study the expression of some defence-related genes. A peroxidase (*CAPO1*), a sesquiterpene cyclase (*CASC1*), a PR1 (*CABPR1*) and a β-1,3-glucanase (*CABGLU*) genes were assayed. Copper stress upregulated the expression of the four genes ranging from 15.5 to 59-fold increase for *CASC1* and *CABPR1*, respectively (Fig. 4). Overall, MCP caused a negligible effect in all the genes and the combination of MCP and copper apparently had no synergic effect, with the values obtained being similar to those obtained with copper alone.

Soluble phenolics, as an important group of antimicrobial compounds, were also determined in stems (Fig. 5). Copper-treated plants showed significantly higher contents of soluble phenolics in the stems than the control plants. The inhibition of ethylene perception with MCP did not itself change the levels of soluble phenolics, but MCP had an antagonistic effect on the phenolic induction caused by copper. When MCP was applied before copper treatment, the increase in soluble phenolics was significantly lower than with copper alone (Fig. 5).

4. Discussion

Many plant diseases have been reported to be influenced by copper, which mainly decreases the disease but in a few cases increases the symptoms [32]. Our results show that pepper plants stressed by copper are less symptomatic when challenged with Verticillium wilt. Copper has direct biocidal effects on micro-organisms, and is a common component of many pesticides, so direct toxicity of the accumulated metal to the pathogen is a likely explanation for the results. However, copper may also play a role in disease resistance because of its involvement in many physiological functions. Copper is a micronutrient that participates in photosynthesis, respiration, antioxidant activity, cell wall metabolism and hormone perception [33]. This essential role of copper in plant physiological processes that influence plant resistance and susceptibility is sometimes overlooked [32]. However, in 1998, Molina et al. [34] demonstrated that in Arabidopsis copper induces resistance against *Peronospora parasitica* in a partially SAR-dependent way. Therefore, a similar copper-induced resistance may have occurred in the present study with pepper plants stressed by copper.

There is evidence that plants respond to abiotic and biotic stresses by the expression of different but overlapping suites of genes [16] and that crosstalk allows plants to regulate both abiotic stress tolerance and disease resistance. Although few studies have focussed on the effect of heavy metal stress on defence against pathogens [6], the present results show that such defence mechanisms are indeed affected. Four defence-related genes, total plant peroxidase activity and total soluble phenolics were induced by copper stress in the present study.

Peroxidases are known to be induced by both abiotic and biotic stresses, including heavy metal stress and pathogen attack [12]. Peroxidases may play several roles in the plant, e.g. in relation to resistance to pathogens. They can produce massive amount of reactive oxygen species (oxidative burst) that are involved in plant cell signalling and also create a highly toxic environment for pathogens. Do et al. [35] suggest that *CAPO1* expression in response to *P. capsici* may be related to ROS-associated defence responses, since peroxidases are closely correlated with H_2O_2 accumulation during the hypersensitive response in resistant cultivars. Recently, another pepper peroxidase gene, *CAPO2*, has been reported to be responsible for H_2O_2 production during the interaction of pepper with bacterial pathogens [36]. On the other hand, peroxidases are also involved in the deposition of cell wall strengthening materials, such as lignin and suberin, which form a mechanical barrier against pathogenic agents. It was previously demonstrated that the induction of peroxidase activity in pepper by copper stress was related to lignin accumulation [9] and that lignification confers tolerance to Verticillium wilt in pepper plants [37]. Moreover, the pepper peroxidase gene *CAPO1* encodes for a basic peroxidase [35], and basic peroxidases have recently been related to the lignification process [38]. The marked increase in *CAPO1* mRNA levels that we observed after 4 days of treatment with copper in pepper stems may be related to participation of this gene in the formation of defensive barriers, although further work would be necessary to confirm this hypothesis. Of course, we cannot rule out the possibility that other peroxidases, e.g. acidic peroxidases, may be involved in the lignification response. Finally, another possible role for copper-induced peroxidases in plant defence is a direct and intrinsic antifungal activity, which has been reported for peroxidases from several plant sources [39–41].

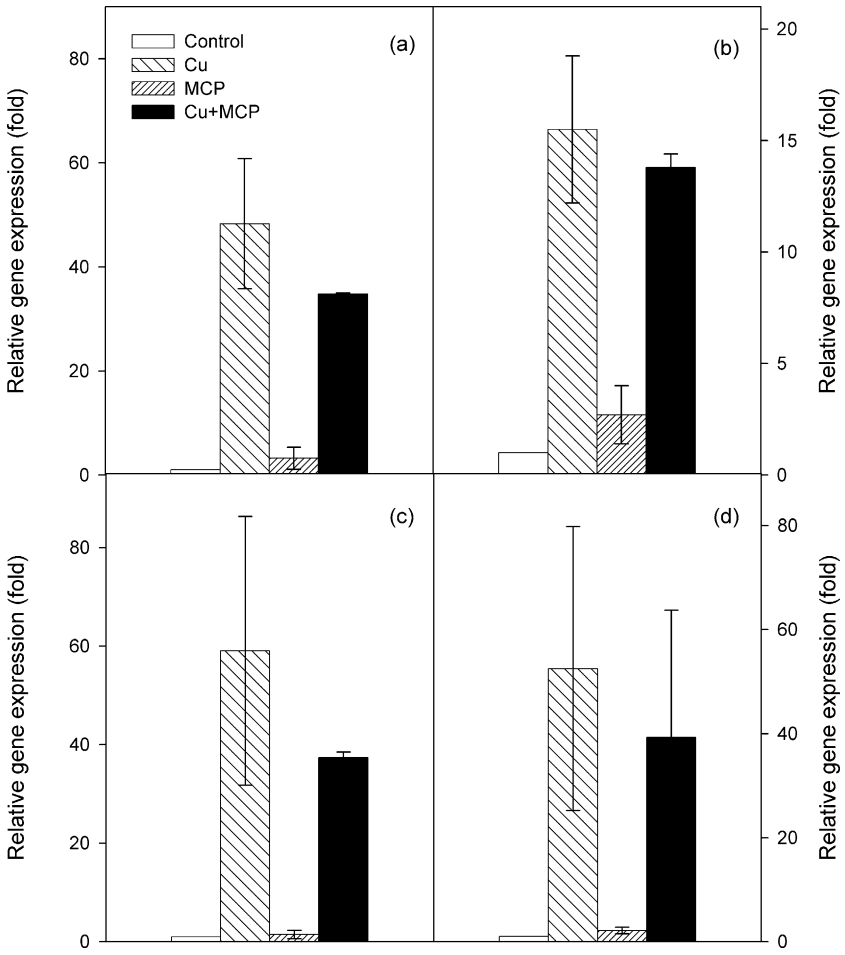

Fig. 4. Effect of copper stress and inhibition of ethylene perception on relative expression levels of defence-related genes in stem of pepper plants. Values are means ± 1S.E. of two independent experiments. (a) *CAPO1*, a peroxidase gene; (b) *CASC1*, a sesquiterpene cyclase gene; (c) *CABPR1*, a PR1 gene and (d) *CABGLU*, a β-1,3-glucanase gene. Co = Control; Cu = 50 μM $CuSO_4$; MCP = 0.2 μL L^{-1} 1-methylcyclopropene; Cu + MCP = 0.2 μL L^{-1} 1-methylcyclopropene + 50 μM $CuSO_4$.

Another common response to biotic stress and heavy metal stress in higher plants is the synthesis and accumulation of defence-related secondary metabolites [13]. Such secondary metabolites include phenolic compounds and terpenoids. We observed an increase in total soluble phenolics in copper-stressed plants, which may be related to copper tolerance [9], but may also influence the response against pathogens. Phenolic compounds have been related to several functions involved in plant defence, namely preformed or inducible physical and chemical barriers against pathogens and local and systemic signalling for the expression of defence genes [42,43]. The accumulation of soluble phenolics in copper-treated hypocotyls may reflect the abundance of intermediate substances in lignin biosynthesis, but also the accumulation of antimicrobial compounds. In pea, copper chloride induces the activity of chalcone synthase, a key enzyme in the biosynthesis of flavonoids, a group of phenolic compounds involved in plant disease resistance [44]. Several phenolic phytoalexins are accumulated in grapevine leaves in response to copper sulphate treatment [45]. In the case of pepper, the main antimicrobial phytoalexin is not a phenolic but a sesquiterpenoid, called capsidiol. Capsidiol is synthesized in the isoprenoid pathway from 5-epi-aristolochene in a reaction catalyzed by a sesquiterpene cyclase with 5-epi-aristolochene synthase activity [46]. The present results showed marked up-regulation of a sesquiterpene cyclase gene, *CASC1*, after 4 days of copper treatment, which may confer a special degree of protection to the plants against the challenge of pathogen attack. This gene is also upregulated as a response to UV light [47] and to *Phytophthora capsici* infection [30], another evidence of crosstalk between abiotic stress and plant response to pathogens.

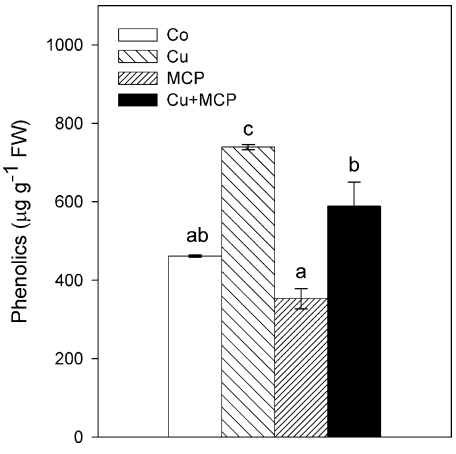

Fig. 5. Effect of copper stress and inhibition of ethylene perception on soluble phenolics in the hypocotyl of pepper plants. Values are means ± 1S.E. of two independent experiments. Co = Control; Cu = 50 μM $CuSO_4$; MCP = 0.2 μL L^{-1} 1-methylcyclopropene; Cu + MCP = 0.2 μL L^{-1} 1-methylcyclopropene + 50 μM $CuSO_4$. The letters above the bars indicate statistically significant differences between groups ($\alpha = 0.05$).

Two other defence-related genes encoding PR proteins, *CABPR1* and *CABGLU*, were also upregulated by copper treatment. The induction of expression of a PR1 gene and a β-1,3-glucanase gene had been reported in tobacco after copper treatment [14]. β-1,3-Glucanases could have a dual role in plant resistance against pathogens, both degrading the pathogen cell wall and releasing oligosaccharides that function as signals for further defence reaction. In the case of PR1 protein, several biological functions have been proposed, but its precise role is still unknown. Several PR1 proteins have anti-oomycete activity, but the mechanisms underlying such an effect remain unknown [48]. Overexpression of *CABPR1* in plants other than pepper, as tomato, tobacco or Arabidopsis confers protection against different pathogens [48–50]. Interestingly, such *CABPR1* overexpression in tobacco causes an increase in tolerance against heavy metals as Hg and Cd [50]. These results and the results presented here suggest a dual role for PR1 both in response to heavy metal stress and defence against pathogens.

All the above-mentioned defences can contribute to resistance against pathogens in copper-stressed plants, but they are the final result of an active signalling in response to the metal stress. Plant hormones as salicylic acid, jasmonic acid and ethylene may also be important in the defence responses observed. Ethylene biosynthesis has been reported to be induced by copper in terrestrial higher plants such as *Phaseolus vulgaris* [23], *Nicotiana tabacum* [24] and *Arabidopsis thaliana* [25]. Moreover, blockage of ethylene perception by Silver Tiosulfate in *Allium cepa* and *Phaseolus coccineus*, lead to an alleviation of copper-induced growth inhibition [21], indicating a role of this hormone in the plant response to the metal. Indeed, after simultaneous application of MCP and copper we observed synergism in several defence responses, such as chitinase and peroxidase activities. Chitinase is induced in rice leaves after 72 h of treatment with 100 μM $CuSO_4$ [51], but in pepper we did not observe any direct induction by copper sulphate alone, perhaps because the dose we used was lower (50 μM $CuSO_4$) or pepper plants are less sensitive. Another possible explanation is the widely reported influence of light on ethylene biosynthesis, which can be positive or negative [26]. Rakwal et al. [51] maintained plants under continuous light after copper treatment and we kept the plants under a 16 h light:8 h dark photoperiod. Rice plants may synthesize less ethylene under continuous light, thus obtaining an effect similar to blockage of ethylene perception. Nonetheless, the present results suggest negative regulation of ethylene on both chitinase and peroxidase activities. It is difficult for us to explain such negative regulation, because it inhibits several responses that may be useful for the plant. For instance, peroxidases play several roles in heavy metal tolerance, such as scavenging free radicals, trapping heavy metals in polymers and degrading toxic molecules [12,52].

It is not clear why pepper plants would under-regulate peroxidase and chitinase by ethylene signalling if these are necessary to resist the effects of copper stress. This is perhaps a matter of feedback regulation. Heavy metal stress induces defence mechanisms and release of ethylene, which modulates the defence response in order to avoid overuse of resources. In any case, the peroxidase *CAPO1* gene was not synergistically upregulated by MCP plus copper in our assays, and the same is true for the other studied plant defence genes, so their regulation could be different. On the other hand, the regulation of soluble phenolics should also be quite different than that suggested for peroxidase activity, because blockage of ethylene partially inhibits phenolic accumulation, both with MCP alone and with MCP plus copper. Ethylene has been involved in the induction of phenylalanine ammonia-lyase, a key enzyme in the biosynthesis of phenolics [53], so it is logical that inhibition of the hormonal action also inhibits biosynthesis of phenols.

In summary, copper induces a plant defence reaction that may cope not only with heavy metal stress but also with plant pathogen attack, and such response could be regulated by plant hormones as ethylene. However, future research is needed to unravel the nature of such regulation.

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